ATP Serves as a Negative Feedback Inhibitor of Voltage-Gated Ca^{2+} Channel Currents in Cultured Bovine Adrenal Chromaffin Cells

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Summary

Modulation of voltage-gated Ca^{2+} channel current (I_{Ca}) regulates secretion of catecholamines from adrenal chromaffin cells. Previous work demonstrated that I_{Ca} can be augmented by phosphorylation to increase secretion or that inhibition of I_{Ca} results in diminished catecholamine secretion. In the current manuscript, we show that stimulation of chromaffin cells results in the release of an "endogenous inhibitor" that suppresses I_{Ca}. The inhibition is due to the secretion of ATP, which is stored at high concentrations in secretory granules and is coreleased with catecholamines upon stimulation. The ATP exerts its actions through P_{2Y} purinoceptors and inhibits both N- and P/Q-type Ca^{2+} channels in a voltage-dependent manner but with different efficacies. Overall, we have identified and characterized a negative feedback pathway that may serve as an important regulatory mechanism for catecholamine secretion in chromaffin cells.

Introduction

Adrenal chromaffin cells secrete catecholamines in response to splanchnic nerve stimulation. The release of acetylcholine (ACh) from the splanchnic nerve terminals activates nicotinic ACh receptors and depolarizes the chromaffin cells leading to increased Ca^{2+} entry (Douglas, 1968). Most of the Ca^{2+} entry is through voltage-gated Ca^{2+} channels (Boarder et al., 1987). Given the nonlinear relationship between intracellular Ca^{2+} levels and secretion (Heinemann et al., 1994), it is likely that modulation of I_{Ca} will have a profound role in the regulation of catecholamine secretion.

Several types of I_{Ca} have been reported in bovine adrenal chromaffin cells including L-, N-, and P/Q-type Ca^{2+} channels (Hoshi and Smith, 1987; Hans et al., 1990; Artalejo et al., 1992a, 1994; Gandía et al., 1993b). The N- and P/Q-type channels are identified pharmacologically by their sensitivity to selective peptide toxins. N-type channels are blocked by ω-conotoxin GVIA, and P/Q-type channels are selectively blocked by low concentrations of ω-agatoxin IVA. The dihydropyridine-sensitive L-type channels, also called facilitation Ca^{2+} channels, are normally quiescent but can be recruited by neurotransmitters like dopamine or vasoactive intestinal peptide (VIP) that elevate intracellular cAMP levels (Wilson, 1988; Artalejo et al., 1990). Alternatively, currents during test depolarizations are greatly potentiated by large predepolarizations or by rapid repetitive depolarization in the physiological range, again by recruitment of L-type facilitation Ca^{2+} channels (Fenwick et al., 1982; Hoshi et al., 1984; Artalejo et al., 1992b). Recruitment of facilitation Ca^{2+} channels augments both Ca^{2+} influx and secretion in chromaffin cells.

We have investigated whether counteracting mechanisms operate to reduce or control Ca^{2+} entry into the chromaffin cells through inhibitory modulation of I_{Ca} by neurotransmitters. It is well established that I_{Ca} can be inhibited in many types of cells by stimulation of G protein–coupled receptors, possibly by direct interaction with the activated G protein (for recent review see Dolphin, 1995). In particular, N-type channels seem to be targeted by inhibitory neurotransmitters, although P-type channels in central neurons are inhibited by similar mechanisms (Bean, 1989; Mintz and Bean, 1993). The inhibition of N-type channels is voltage dependent, since there is relief from block at positive test potentials or after a prepulse to a very positive potential (Bean, 1989; Elmslie et al., 1990; Penington et al., 1991). There is also a characteristic slowing of activation in the presence of an inhibitory neurotransmitter. Many of these characteristics have been successfully described using a model in which the Ca^{2+} channels exhibit two functional gating states; one in the presence and another in the absence of inhibition (called "reluctant" and "willing" gating states, for example see Bean, 1989).

Chromaffin cell secretory granules contain a number of neurotransmitters in addition to catecholamines (Carmichael and Winkler, 1985; Winkler et al., 1988), many of which have been demonstrated to inhibit I_{Ca} in other cell types. One or several of these neurotransmitters act on autoreceptors located on the chromaffin cells, as it has been reported that I_{Ca} is inhibited by release of an endogenous substance from the cells maintained in a static bath under depolarizing conditions. Superfusion of the cell resulted in an increase of I_{Ca} due to washout of the inhibitor (Doupnik and Pun, 1994). It is probable that the "endogenous inhibition" represents a negative feedback mechanism in which transmitters released from the chromaffin cells act in an autocrine/paracrine fashion to inhibit I_{Ca} and control secretion.

There have been several previous reports of exogenously applied transmitter-mediated inhibition of I_{Ca} in chromaffin cells. These include inhibition by D_2 dopamine receptors (Bigornia et al., 1990), γ-aminobutyric acid type B ($GABA_B$) receptors (Doroshenko and Neher, 1991), α_2-adrenergic receptors (Kleppisch et al., 1992), and opioid receptors (Kleppisch et al., 1992; Twitchell and Rane, 1993). ATP has been shown to inhibit I_{Ca} in chromaffin cells through a G protein–mediated pathway (Diverse-Pierluissi et al., 1991; Gandía et al., 1993a) that is of particular interest since ATP is stored in chromaffin cell secretory vesicles at a high concentration (Winkler and Westhead, 1980).

In this study, we have investigated the action on I_{Ca} of both endogenous inhibitors released from the chromaffin cells upon stimulation and of exogenously applied neurotransmitters. To study the voltage dependence of the inhibition of I_{Ca}, we employ depolarizing prepulses similar to those used to recruit facilitation Ca^{2+} channels; therefore, we have chosen to block the

facilitation Ca^{2+} channels (L-type) with nisoldipine. This allows investigation of endogenous inhibition and its voltage dependence. We show that stimulating chromaffin cells results in the secretion of an endogenous inhibitor that suppresses I_{Ca}. Furthermore, of nine neurotransmitters tested, only opioids and ATP were shown to inhibit I_{Ca}. Most interestingly, we demonstrate that the endogenous inhibition of I_{Ca} in chromaffin cells is due to release of ATP from the secretory granules that acts through P_{2Y} receptors to inhibit I_{Ca} in a voltage-dependent manner. Both N- and P/Q-type components of I_{Ca} are suppressed but with different efficacies. In contrast with earlier studies (Doupnik and Pun, 1994), our results show that the increase in I_{Ca} observed due to relief of block by prepulses is mechanistically different than recruitment of facilitation, as we have previously shown that facilitation Ca^{2+} channels are blocked by nisoldipine (1 μM) (Artalejo et al., 1992b, 1994) and we have included nisoldipine in all of our external solutions in the current study. Overall, the data identify and characterize a negative feedback control mechanism for the regulation of catecholamine secretion from adrenal medullary chromaffin cells.

Results

Chromaffin Cells Secrete an Endogenous Inhibitor That Suppressed I_{Ca}

To investigate the effects of endogenous transmitters secreted from the chromaffin cells, we exposed a plate of cells to a tetraethylammonium (TEA)/Ba-containing extracellular solution. This solution depolarized the chromaffin cells and triggered secretion in every cell except for the one that was voltage clamped. With the bath solution rapidly flowing, any secreted material was quickly washed away, but when the bath flow was stopped, the secreted endogenous transmitters accumulated in the bath at a level sufficient to exert an action on I_{Ca} (see Experimental Procedures). In contrast with the depolarizing solution, a NaCl/Ca-based recording solution (see Experimental Procedures) did not depolarize the non-voltage-clamped cells or trigger secretion.

Peak inward I_{Ca} were activated by 20 ms step depolarizations from −80 mV to +10 mV every 20 s with the bath solution flowing or with the flow stopped. With the cells bathed in the NaCl/Ca-based extracellular recording solution, stopping the flow of the bath solution had no action on I_{Ca} (n = 9, Figure 1A). However, when the cells were bathed in the TEA/Ba-based recording solution, stopping the flow caused a reversible inhibition of I_{Ca} in virtually every cell tested (59 out of 61 cells, Figure 1A). In all cases, the inhibition was quickly reversed when the flow of bath solution was resumed. Endogenous inhibition could be elicited several times in every responsive cell by stopping and then starting the bath flow multiple times (data not shown). The extent of the inhibition of peak I_{Ca} ranged from approximately 10%–50% with a mean inhibition of 29% ± 2% (n = 59). In addition to suppressing the peak current amplitude, the endogenous inhibition also slowed the activation kinetics of I_{Ca} and was voltage dependent (Figure 1),

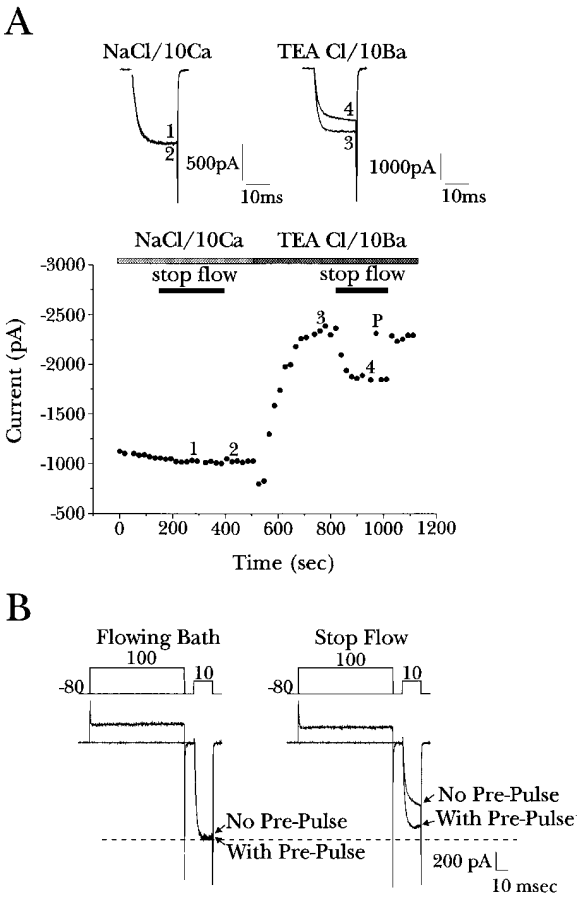

Figure 1. Chromaffin Cells Release an Endogenous Inhibitor of I_{Ca} When a Depolarizing Solution (TEA/Ba) Is Introduced into the Bath but Not When a NaCl/Ca-Based Solution Is Present

(A) Plots peak inward I_{Ca} amplitude as a function of time. Each point represents the peak amplitude elicited by a step depolarization from −80 mV to +10 mV. The numbered points correspond to the current records shown above. A prepulse to +100 mV (100 ms) was applied at the point labeled with a P. Currents 1 and 2 shown above were recorded with the solution stopped and flowing, respectively, in the NaCl/Ca-based bath solution. Currents 3 and 4 were recorded with the TEA/Ba solution flowing and stopped, respectively.

(B) Current records from a second cell bathed in the TEA/Ba-based recording solution showing that the block by the endogenous inhibitor was relieved by depolarizing prepulses. On the left are shown two superimposed currents recorded with the bath solution flowing. The upper traces show the voltage protocols used to activate I_{Ca}. One current (no prepulse) was activated by a step depolarization from −80 mV to +10 mV and the second current (with prepulse) was activated in the same way except that it was preceded by a depolarizing prepulse to +100 mV. The traces to the right were obtained under similar conditions except that the flow of the bath solution was stopped. The dashed line is for illustrative purposes and corresponds to the peak amplitude of I_{Ca} in control conditions with the bath solution flowing.

consistent with previous reports of neurotransmitter inhibition of I_{Ca} (Mintz and Bean, 1993; Hille, 1994). A depolarizing prepulse to +100 mV applied for 100 ms, 10 ms prior to the test pulse, relieved between 60%–80% of the inhibition and also sped up the activation rate of I_{Ca}. The same voltage protocol had little or no effect on control currents recorded with the bath solution flowing

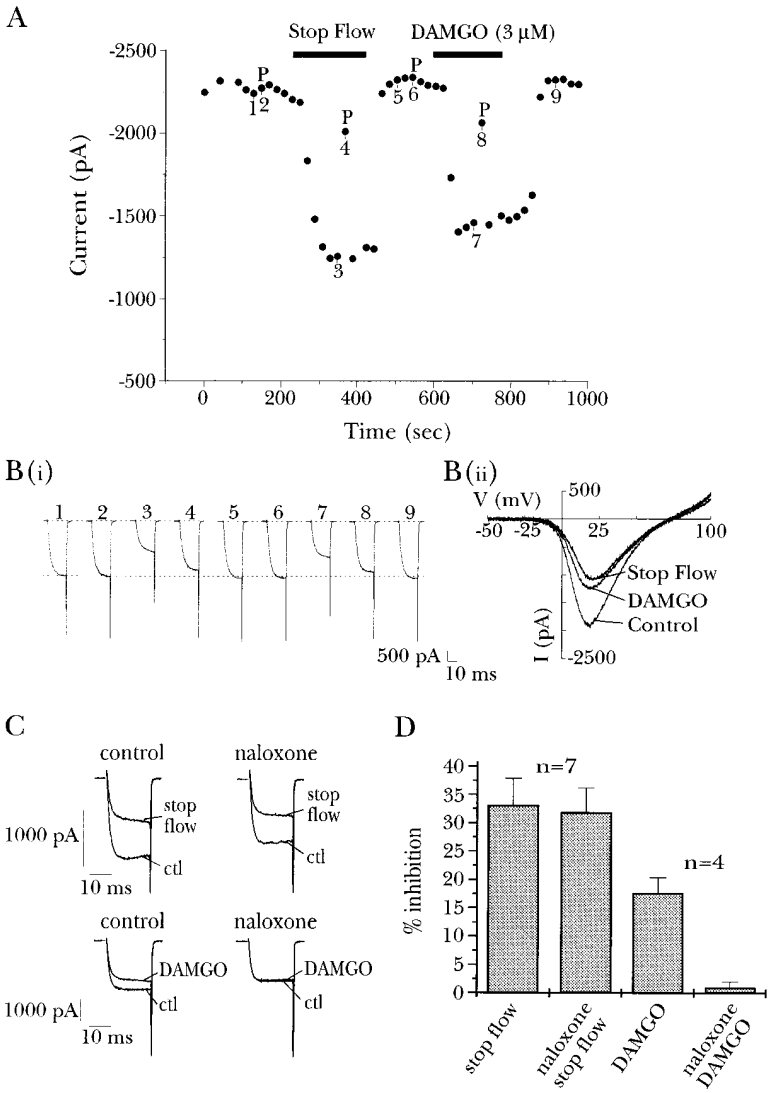

Figure 2. The μ-Opioid Receptor Agonist DAMGO Inhibits I_{Ca} in Chromaffin Cells but the Opioid Receptor Antagonist Naloxone Does Not Block Endogenous Inhibition

(A) Peak inward I_{Ca} amplitude is plotted against time. Currents were activated every 20 s by step depolarizations with the bath solution flowing. The numbered points correspond to the current records shown in (B[i]). Those currents represented by the points labeled P were preceded by depolarizing prepulses to +100 mV like those shown in Figure 1B. Endogenous inhibition was elicited by stopping the bath flow as indicated. Application of 3 μM DAMGO is indicated by the horizontal bar.

(B[ii]) Current–voltage relationships from the same cell recorded under control conditions, with the flow of the bath solution stopped to elicit endogenous inhibition (labeled stop flow) and during 3 μM DAMGO perfusion. Peak inhibition was observed at +20 mV with little or no inhibition at potentials positive to +50 mV. I/V curves were generated by voltage ramps of 100 ms duration from −80 mV to +100 mV and have been leak subtracted.

(C) Currents recorded from two cells in control conditions (with no antagonist) and with 10 μM naloxone present. The upper traces show currents with the bath solution flowing (ctl) and with the flow of solution stopped (stop flow) to elicit endogenous inhibition. The presence of naloxone did not prevent endogenous inhibition. In contrast, the inhibition produced by DAMGO (1 μM) was totally blocked by the presence of naloxone (lower traces).

(D) Bar chart showing the mean percent inhibition of peak inward I_{Ca} by the endogenous inhibitor (stop flow), by the endogenous inhibitor with 10 μM naloxone present (naloxone stop flow), by 1 μM DAMGO (DAMGO), and by 1 μM DAMGO with 10 μM naloxone present (naloxone DAMGO). Standard error bars and number of cells (n) are shown.

(Figure 1B). These results indicate that an endogenous inhibitor was released from non-voltage-clamped chromaffin cells upon exposure to the TEA/Ba-based solution.

The endogenous inhibition was pertussis toxin (PTX) sensitive, since preincubation of the cells with 100–300 ng/ml PTX for 18–40 hr virtually abolished the inhibition. In control cells from the same culture and recorded on the same day as the PTX-treated cells, the extent of the endogenous inhibition was 22% ± 7% (n = 7), but was only 4% ± 1% (n = 9; $p \leq 0.01$) in PTX-treated cells. The amplitude of I_{Ca} was unaltered by PTX treatment, being 1800 ± 180 pA (n = 7) in control cells and 1760 ± 160 (n = 9) in PTX-treated cells.

Most Neurotransmitters Tested Did Not Inhibit I_{Ca}

In addition to catecholamines, chromaffin cells have also been reported to contain a wide variety of neurotransmitters (Carmichael and Winkler, 1985; Winkler et al., 1988), many of which are known to modulate I_{Ca}. To try and uncover the identity of the endogenous inhibitor, various neurotransmitters or agonists were washed into the bath to see whether they would mimic the suppression of I_{Ca} produced by the endogenous inhibitor. Most neurotransmitters or agonists tested had no action on I_{Ca}. These included norepinephrine and the selective α_2-adrenergic agonist UK 14304 (n = 5), the β-adrenergic agonist isoproterenol (n = 3), the D_2-dopaminergic agonist quinpirole (n = 3), serotonin (n = 4), the $GABA_B$ agonist baclofen (n = 4), neuropeptide Y (n = 4), and the adenosine receptor agonist 2-chloro-adenosine (n = 4).

Opioid Receptor Activation Inhibited I_{Ca}

In contrast with the neurotransmitters tested above, the μ-opioid receptor agonist [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) did inhibit I_{Ca} (Figure 2). Most cells tested (21 out of 23) showed an inhibition of peak inward I_{Ca} when DAMGO (1–3 μM) was applied. The inhibition exhibited the same characteristics as the endogenous inhibition in that it was reversible, voltage dependent, and accompanied by a slowing of the activation kinetics of I_{Ca} (Figures 2A and 2B). The peak inward I_{Ca} was inhibited by 26% ± 6% (n = 5) and 27% ± 3% (n = 7) by 1 and 3 μM DAMGO, respectively, while in the same set

of cells the endogenous inhibition was 36% ± 4% (n = 11). The inhibition produced by DAMGO was not additive to the endogenous inhibition, since the suppression of peak I_{Ca} produced by stopping the flow of the bath solution in the absence and presence of DAMGO was identical (39% ± 5% and 39% ± 6%; n = 4), indicating that the same pool of channels were inhibited and opening the possibility that the endogenous inhibition may be due to release of opioids.

The Opioid Receptor Antagonist Naloxone Did Not Block Endogenous Inhibition

After establishing the presence of endogenous inhibition, the cells were washed with a solution containing 10 μM naloxone for several minutes and then the flow of the bath solution was stopped a second time to elicit endogenous inhibition. The endogenous inhibition was found to be unaltered by the naloxone (Figures 2C and 2D), being 33% ± 5% in the absence and 32% ± 4% in the presence of the antagonist (n = 7). In 4 of these cells, DAMGO was also tested, and it was found that the naloxone abolished the DAMGO-induced inhibition (Figures 2C and 2D). So although opioid receptor activation inhibited I_{Ca}, opioids were not responsible for endogenous inhibition.

ATP Mimicked Endogenous Inhibition

Bovine adrenal chromaffin cells are known to contain a substantial amount of ATP and other nucleotides in their secretory granules (Winkler and Westhead, 1980). As ATP has previously been reported to inhibit I_{Ca} in these cells (Gandía et al., 1993a), we tested ATP to see whether it would mimic endogenous inhibition. ATP reversibly inhibited I_{Ca} in a voltage-dependent manner and produced a slowing of activation kinetics (Figure 3). Most cells tested (36 out of 40) responded to ATP. The mean percentage inhibition produced by 10 μM ATP was 24% ± 3% (n = 15) and by 100 μM ATP was 31% ± 4% (n = 9). In the same group of cells, the endogenous inhibitor produced a block of 34% ± 2% (n = 26). The ATP-induced inhibition was not additive with the endogenous inhibition. In control conditions, the inhibition was 27% ± 3% (n = 6), and with 10 μM ATP present, it was identical at 27% ± 3% (n = 6).

Characterization of the ATP Response

To prevent secretion from the non-voltage-clamped cells, these experiments were performed in NaCl-based recording medium with 10 mM Ca^{2+} as the charge carrier. Tetrodotoxin was included in the medium to suppress the Na^+ current, and the K^+ current was efficiently suppressed by the intracellular Cs^+ in the patch pipette solution. ATP was applied as before. Figure 4A shows the dose–response curve for ATP obtained under these conditions, which gave an EC_{50} of about 0.5 μM. Like endogenous inhibition, the ATP-induced inhibition of I_{Ca} was PTX sensitive. Cells preincubated for 18–40 hr with 300 ng/ml PTX were compared with untreated control cells of the same age from the same cultures. In control cells, 100 μM ATP produced an inhibition of 50% ± 7% (n = 4), while in PTX-treated cells, 100 μM ATP caused only 3% ± 2% inhibition of I_{Ca} (n = 4). This confirmed

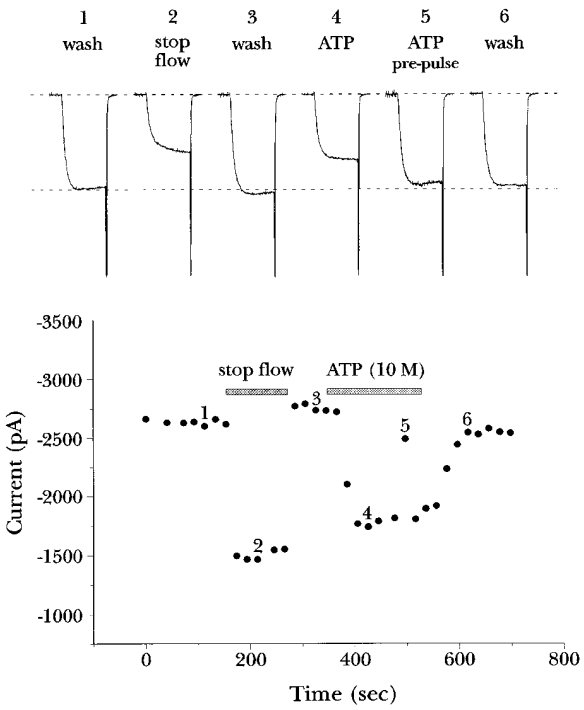

Figure 3. ATP Mimics Endogenous Inhibition

Peak inward I_{Ca} amplitude is plotted against time. The numbered points correspond to the current records shown above. Stopping the bath flow caused an endogenous inhibition of I_{Ca}. This was mimicked by perfusion of 10 μM ATP (indicated by a horizontal bar). The ATP-induced inhibition was voltage dependent, as it could be almost completely relieved by a depolarizing prepulse (trace number 5). Current traces 1–6 were elicited by depolarization to +10 mV from a holding potential of −80 mV. The depolarization in trace 5 was preceded by 100 ms prepulse to +100 mV, followed by a 10 ms return to the holding potential.

that ATP was acting through a PTX-sensitive G protein–linked receptor.

The pharmacology of purinergic receptors is somewhat confusing, as there are no selective antagonists for the various receptor subtypes. Typically, relative agonist potencies have been used for receptor identification. To try and identify which receptor was responsible for the ATP inhibition of I_{Ca} in chromaffin cells, various P_2 agonists were tested. Equimolar concentrations of the agonists and ATP itself were tested on the same cell, and the percentage inhibition produced by the agonist in question was normalized with respect to the percentage inhibition produced by ATP. Pooled data revealed the order of activity at 10 μM was 2-methylthio-ATP (1.35 ± 0.18; n = 4) = ADP (1.34 ± 0.18; n = 4) ≥ ATP (1) >> UTP (0.34 ± 0.13; n = 5), and diadenosine tetraphosphate (Ap4A) was found to produce no inhibition even at 100 μM (n = 3) (the numbers in brackets following each agonist represent the mean normalized response with respect to ATP). This rank order of activity is consistent with ATP acting at a P_{2Y} subtype of purinergic receptor (Dalziel and Westfall, 1994; Fredholm et al., 1994).

Both the trypanocidal drug suramin and the anthraquinone–sulfonic acid derivative reactive blue-2 (RB-2)

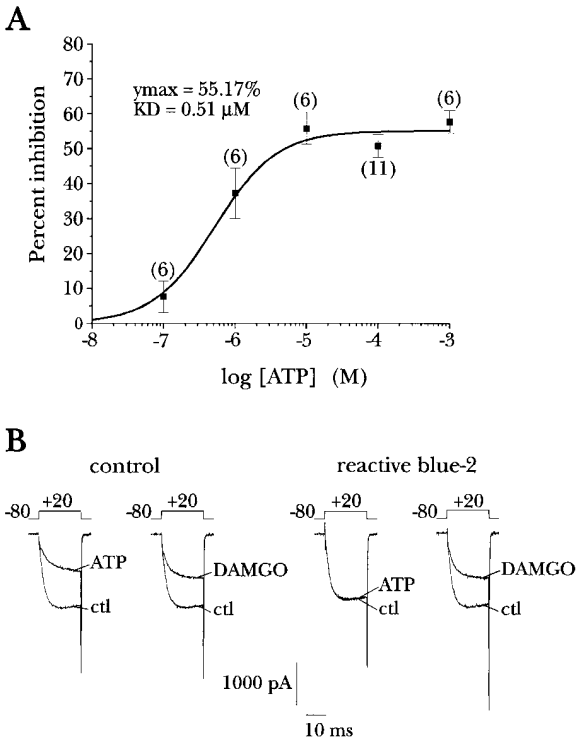

Figure 4. ATP Dose–Response Relation for I_{Ca} Inhibition

(A) Log_{10} [dose] of ATP is plotted as a function of I_{Ca} inhibition. Data is from cells bathed in NaCl-based recording medium. The mean percent inhibition of peak inward I_{Ca} produced at each concentration of ATP is plotted. The number of cells are indicated beside each data point along with standard error bars. The data were fit by a curve defined by the function $Y = Y_{max} \times 1/1 + (K_D/X)$, in which Y is the percent inhibition and X is the ATP concentration.

(B) Shows current records all recorded from the same cell, demonstrating that 30 μM RB-2 blocks the ATP-induced inhibition of I_{Ca} but has no effect on the DAMGO-induced inhibition. The voltage protocol used to activate the currents is shown above each trace. The currents on the left were recorded under control conditions (with no RB-2 present) with the bath flowing and no agonist present (ctl) or with either 100 μM ATP (ATP) or 3 μM DAMGO (DAMGO) present. The currents on the right were recorded under the same conditions except that RB-2 was present and clearly show that DAMGO inhibited I_{Ca} to the same extent, whereas the ATP-induced inhibition was abolished.

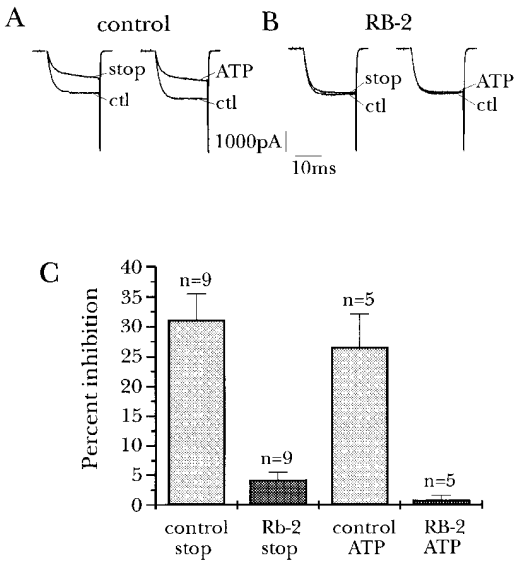

Figure 5. RB-2 Blocks Endogenous Inhibition of I_{Ca}

(A) Shows current records from a cell bathed in TEA/Ba-based recording medium. In each case, two currents are superimposed. Currents were recorded with the bath solution flowing (ctl), with the flow stopped to elicit an endogenous inhibition (stop), or with the solution flowing but containing 10 μM ATP (ATP). This cell showed a robust inhibition of I_{Ca} produced by both the endogenous inhibitor and ATP.

(B) Shows currents from the same cell as in (A) except that there was also 30 μM RB-2 present. Both the endogenous inhibition produced by stopping the flow of the bath solution (stop) and the ATP induced inhibition were abolished. All currents in (A) and (B) were activated by step depolarizations from −80 mV to +20 mV.

(C) Bar chart showing pooled data from several cells. Plot of the mean percent inhibition of peak inward I_{Ca}. Standard error bars are shown along with the number of cells (n). Shown are the endogenous inhibition produced by stopping the flow of the bath solution in the absence(control stop) and presence of RB-2 (RB-2 stop) and also the inhibition produced by ATP in the absence (control ATP) and presence of RB-2 (RB-2 ATP).

have been used as P_2 purinergic receptor antagonists, although both drugs possess only limited selectivity (Dalziel and Westfall, 1994; Fredholm et al., 1994). Of the two antagonists, RB-2 seems to exhibit a somewhat higher degree of selectivity for the P_{2Y} receptor than does suramin (although this may vary between species and tissue type), so this putative P_{2Y} receptor antagonist was tested on the ATP response in chromaffin cells. RB-2 had no direct action on the Ca^{2+} current, which had amplitudes of 1330 ± 210 pA (n = 5) and 1290 ± 210 pA (n = 5) in the absence and presence of 30 μM RB-2, respectively. The mean control inhibition elicited by ATP application in the absence of RB-2 was 45% ± 5% (n = 6). With 3 μM RB-2 present, only 2 out of 3 cells showed any inhibition (6% ± 3%: n = 2), and with 30 μM RB-2 present, only 1 out of 3 cells showed an inhibition (6%; n = 1). In contrast, 30 μM RB-2 did not block the inhibition produced by 3 μM DAMGO, which was 39% ± 10% (n = 3) in the absence and 45% ± 6% (n = 3) in the presence of RB-2.

RB-2 Blocked the Endogenous Inhibition

Since RB-2 was an effective antagonist of the ATP-mediated inhibition of I_{Ca}, its effects on endogenous inhibition were investigated. The TEA/Ba-based recording medium was employed in these experiments. After obtaining and reversing endogenous inhibition by stopping and then restarting the flow of the bath solution, the cells were washed with 30–100 μM RB-2 for several minutes before the flow was stopped a second time. In all cases, the endogenous inhibition was almost completely abolished by RB-2 (Figure 5). The mean percentage inhibition under control conditions was 31% ± 4% (n = 9); in the same cells with RB-2 present, only a small residual inhibition of 4% ± 1% ($p \leq 0.001$) was observed. In 5 cells tested, RB-2 also abolished the inhibition produced by applying ATP directly to the cells ($p \leq 0.01$) (see Figure 5), but the inhibition produced by

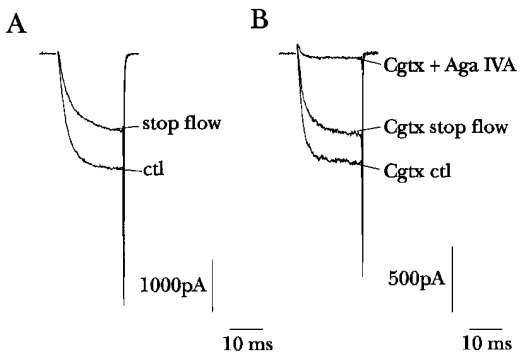

Figure 6. Both N- and P/Q-Type I_{Ca} Are Suppressed by the Endogenous Inhibition

(A) The larger current was activated with the bath solution flowing (ctl) and the second (smaller) current was activated with the flow of solution stopped to elicit endogenous inhibition (stop flow).

(B) Currents are from the same cell shown in (A), but note the change in the scale bar. All currents were recorded after the cell had been exposed to ~1 μM ω-conotoxin GVIA (Cgtx) to selectively block N-type channels. The largest current was recorded with the bath solution flowing (Cgtx ctl), which shows that over 50% of the current was irreversibly blocked by Cgtx. When the bath flow was stopped, the current was inhibited (Cgtx stop flow) to a lesser extent, indicating that both the N-type and Cgtx-resistant currents were suppressed by the endogenous inhibitor. The cell was then exposed to ~100 nM ω-agatoxin IVA, which selectively blocks P/Q-type I_{Ca}, to demonstrate that almost all the remaining current is P/Q-type (Cgtx + Aga IVA).

DAMGO application was unaltered (n = 2). These results suggest that the endogenous inhibition was due to release of ATP from the chromaffin cells.

Both N- and P/Q-Type I_{Ca} Were Suppressed by the Endogenous Inhibition

Several types of I_{Ca} have been reported in cultured bovine adrenal chromaffin cells. In this study, L-type facilitation Ca^{2+} channels were blocked by including 1 μM nisoldipine in all the extracellular solutions. The remaining current was almost totally accounted for by N- and P/Q-type channels (see Figure 6). N-type I_{Ca} was identified as a current component blocked by ω-conotoxin GVIA (Cgtx), and P/Q-type current was identified as the component blocked by ω-agatoxin IVA (Aga). The mean percentage inhibition of peak I_{Ca} by Cgtx was 50% ± 3% (n = 9) and by Aga was 46% ± 2% (n = 7) leaving a small component, 4% ± 1% (n = 7), resistant to both blockers, consistent with previously published work (Artalejo et al., 1994).

To determine whether both the N- and P/Q-type I_{Ca} were suppressed, endogenous inhibition was elicited in the absence and presence of Cgtx. Cgtx was added directly to the bath with the solution flow stopped. The flow was then restarted, but the N-type channels remained blocked. With no Cgtx present, the endogenous inhibition was 32% ± 5% (n = 9), and in the same cells with Cgtx present, it was significantly less at 17% ± 3% (p = 0.001). Assuming that maximal amounts of ATP were released into the bath before and after Cgtx application, then the data suggest that both the N- and P/Q-type currents were suppressed by the endogenous inhibitor but that the N-type channels were inhibited to a greater extent.

ATP Inhibition of I_{Ca} Was Unaffected by Intracellular Ca^{2+} Buffering

Neurotransmitter modulation of N-type I_{Ca} has been studied in considerable detail, and five different signaling pathways have been identified for various transmitters (Hille, 1994). These include several membrane delimited pathways and a diffusible pathway that is sensitive to intracellular Ca^{2+} buffering. In addition, it is known that in many cell types P_{2Y} purinergic receptors are coupled to phospholipase C (Boarder et al., 1995) and can elevate intracellular Ca^{2+} by release of IP_3 sensitive stores, which may in turn suppress I_{Ca}. To determine whether these pathways were involved in the ATP inhibition of I_{Ca} in the chromaffin cells, experiments were performed using patch pipette solution that contained either 10 mM EGTA (the patch pipette solution used in all the data reported above) or 0.1 mM EGTA. The cells were bathed in the NaCl-based recording medium, and the inhibition produced by ATP showed no significant differences under the two recording conditions (Figure 7). The mean percentage inhibition of peak inward I_{Ca} was 51% ± 3% (n = 11) with 10 mM EGTA and 50% ± 4% (n = 6) with 0.1 mM EGTA (Figures 7A, 7B, and 7C). There was also no significant difference in the percentage of this inhibition that was reversed by a 100 ms depolarizing prepulse to +100 mV applied 10 ms prior to the test pulse. With 10 mM EGTA, the percentage of inhibition relieved by the prepulse was 73% ± 3% (n = 9), and with 0.1 mM EGTA, it was 70% ± 4% (n = 6) (Figures 7A, 7B, and 7D). The actual current amplitudes recorded from cells using the 0.1 mM EGTA patch pipette solution were slightly smaller, but the mean amplitudes were not significantly different, being 1170 ± 120 pA (n = 11) and 980 ± 200 pA (n = 6) for the 10 mM and 0.1 mM EGTA solutions, respectively.

Discussion

It has been shown previously that augmentation of Ca^{2+} influx by recruitment of facilitation Ca^{2+} channels can accelerate secretion, suggesting that this mechanism may play a role in the "fight or flight" response in which a large increase of catecholamine secretion is required (Artalejo et al., 1994). It seems likely that opposing mechanisms exist which act to reduce catecholamine secretion by down-regulating I_{Ca}. Indeed, it has been reported that stimulation of chromaffin cells results in the release an inhibitor of I_{Ca} (Doupnik and Pun, 1994). In our current study, we have also found that chromaffin cells release an endogenous inhibitor of I_{Ca}, but we show that this process is distinct from facilitation, as all of our studies were carried out in the presence of 1 μM nisoldipine, which was previously shown to inhibit L-type facilitation Ca^{2+} channels (Hoshi and Smith, 1987; Artalejo et al., 1990, 1994). Thus, the increases in I_{Ca} reported here produced by prepulses represent relief of inhibition not recruitment of facilitation current. In addition, we characterize the endogenous inhibition ultimately demonstrating that it is due to release of ATP from the chromaffin cells.

The endogenous inhibitor suppressed I_{Ca} in a PTX-sensitive, voltage-dependent manner slowing the activation kinetics and showing relief of inhibition at depolarized potentials or after prepulses to +100 mV. In some

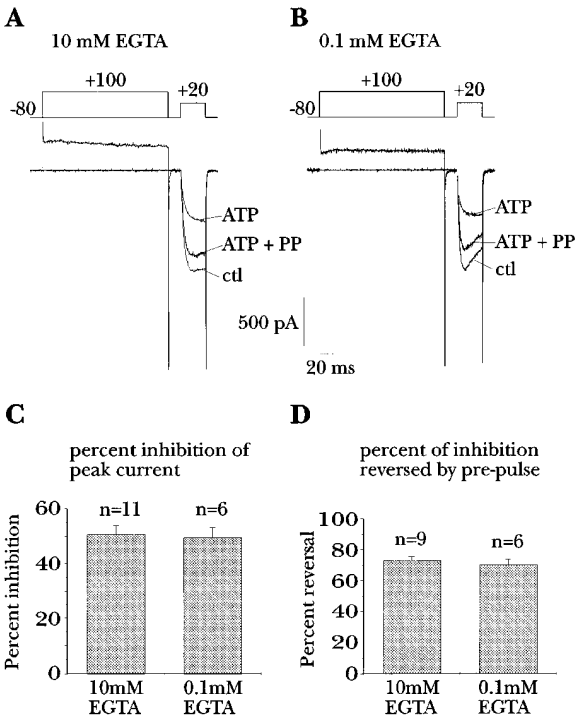

Figure 7. The Inhibition Produced by ATP Is Not Dependent on Intracellular Ca^{2+} Buffering

The NaCl/Ca-based bath solution was used in these experiments.

(A) Current records from a cell with 10 mM EGTA present in the patch pipette solution. The upper traces show the voltage protocols used to activate I_{Ca}. The largest I_{Ca} (ctl) shows the current elicited with no ATP present, while the smallest current was obtained in the presence of 100 μM ATP. The third current (ATP + PP), also recorded in the presence of 100 μM ATP, was preceded by a depolarizing prepulse to +100 mV.

(B) Is of the same format as the data presented in (A), except that the currents were recorded from a cell with 0.1 mM EGTA present in the patch pipette solution. Note that the ATP-induced inhibition and prepulse reversal of this inhibition appear to be very similar in the two cells. The scale bar applies to both (A) and (B).

(C) Bar chart of pooled data showing the mean percent inhibition of peak inward I_{Ca} by 100 μM ATP was virtually identical in cells recorded with patch pipette solution containing either 10 mM or 0.1 mM EGTA. Standard error bars and number of cells (n) are shown and statistical analysis showed no significant difference.

(D) Bar chart of pooled data showing the voltage dependence of the inhibition produced by 100 μM ATP is virtually identical in cells recorded with patch pipette solution containing 10 mM or 0.1 mM EGTA. The Y axis (percent reversal) represents the ATP inhibition reversed by a depolarizing prepulse as shown in (A) and (B). Statistical analysis showed no significant difference for cells recorded under the two conditions.

cells, I_{Ca} was inhibited even with the bath solution flowing (data not shown), as demonstrated by relatively slow activation and an increase in the amplitude and speeding of the activation kinetics of I_{Ca} by a prepulse to +100 mV. This was only seen in cells bathed in the TEA/Ba-based recording medium and never seen in the NaCl/Ca-based recording medium. The partial block was attributed to incomplete washout of secreted material from around the voltage-clamped cell, even with the bath flowing. Often this "tonic" inhibition would wash out partly or wholly after time. It should also be pointed out that the mean percentage inhibition of peak inward I_{Ca} produced by both DAMGO and ATP were greater in cells bathed in the NaCl-based recording medium rather than the TEA/Ba-based recording medium (45%–55% versus 25%–30%). Part of the difference may be due to tonic inhibition in some cells in the TEA/Ba-based recording medium.

Previous reports have concluded that I_{Ca} can be inhibited in chromaffin cells by activation of D_2-dopaminergic (Bigornia et al., 1990), α_2-adrenergic (Kleppisch et al., 1992), or $GABA_B$ receptors (Doroshenko and Neher, 1991), but none of these suppressed I_{Ca} in this study. In the report of α_2-adrenergic modulation, it was concluded that L-type channels were inhibited; these channels were blocked with nisoldipine in the current study. The reasons for the disparity in the D_2 and $GABA_B$ responses are not clear, but may be due to differences in the age of animals used to prepare the cell cultures, selection of a certain subpopulation of cells, or modulation of L-type channels. Chromaffin cells store and secrete opioids and ATP (Winkler and Westhead, 1980), and our data do concur with reports that both opioids (Kleppisch et al., 1992; Twitchell and Rane, 1993) and ATP (Diverse-Pierluissi et al., 1991; Gandía et al., 1993a) inhibit I_{Ca} in chromaffin cells. Closer analysis of the ATP response revealed that it was PTX sensitive, voltage dependent, and mediated by the activation of P_{2Y} purinoceptors. The putative P_2 receptor antagonist RB-2 (which shows a certain degree of selectivity for P_{2Y} receptors in some cell types) blocked both the ATP-mediated inhibition and the endogenous inhibition of I_{Ca} in the chromaffin cells, but had no effect on the DAMGO-induced inhibition. In contrast, the endogenous inhibition was unaffected by application of the opioid receptor antagonist naloxone. Taken together, these data show that endogenous inhibition is due to release of ATP, but not opioids from the chromaffin cells. In our recording conditions, the concentration of released opioid may not have been sufficient to activate μ-receptors. In the more restricted diffusional space of an intact adrenal gland, opioids may accumulate sufficiently to produce receptor activation. μ-Opioid receptor activation has also been shown to potentiate large conductance Ca^{2+}-activated K^+ channel (BK) currents (Twitchell and Rane, 1993, 1994). It may be that the effects on I_{Ca} are secondary, with the main target of opioid action being the BK channels.

Using long depolarizing stimuli, Lopez et al. (1994) showed that blocking N-type Ca^{2+} channels had little effect on secretion, presumably because during these long depolarizations sufficient Ca^{2+} enters via other Ca^{2+} channels to maximally activate secretion. Assuming that comparable amounts of endogenous inhibitor were released before and after application of Cgtx, our data show that the endogenous inhibitor suppressed both the N- and P/Q-type channels, but the N-type was inhibited to a greater extent. There has been little direct comparison of neurotransmitter-mediated inhibition of N- and P/Q-type I_{Ca}, although in spinal cord neurons both are modulated by GABA in a similar fashion except the N-type channels are inhibited to a slightly greater extent (Mintz and Bean, 1993). The mechanistic explanation for the differential inhibition of the two channel types remains to be determined.

Several pathways distinguished by voltage dependence, PTX sensitivity, and sensitivity to intracellular Ca^{2+} buffers have been identified for neurotransmitter modulation of I_{Ca} (for review see Hille, 1994). Based on the similarity of our data recorded from cells with either 0.1 mM or 10 mM EGTA in the patch pipette solution, we can rule out involvement of the diffusible, Ca^{2+} buffer–sensitive pathway in the ATP inhibition of I_{Ca} in chromaffin cells.

This paper reports a role for ATP as an inhibitory modulator of secretion in chromaffin cells that is in contrast with some previous reports that ATP can stimulate secretion from adrenal chromaffin cells via activation of P_{2X} purinoceptors (Lin et al., 1995). P_{2X} receptors, several of which were recently cloned (Brake et al., 1994; Valera et al., 1994), are ligand-gated ion channels similar to nicotinic ACh receptors in that they are relatively nonselective, allowing flux of Na^+, K^+, and Ca^{2+} (Bean, 1992), and will thus depolarize the cell upon activation. We did not observe any evidence of ATP-gated inward currents activated under our recording conditions, although we were not specifically looking for such currents and so the experimental setup was not optimized for their detection. The ATP was applied slowly to the cells and so any responses that desensitized rapidly, as has been reported for some but not all P_{2X} receptors (Bean, 1992; Evans et al., 1995), may not have been detected. There is some evidence to suggest these P_{2X} receptors are preferentially localized on norepinephrine-containing cells (Castro et al., 1995; Lin et al., 1995), whereas our cultures were somewhat enriched for epinephrine-containing cells.

Although not the focus of this study, it is interesting to consider what effect if any ATP might exert on L-type facilitation Ca^{2+} channels. These channels are recruited by transmitters which elevate intracellular cAMP levels. In most systems studied, activation of P_{2Y} receptors is coupled to stimulation of phospholipase C (Dubyak and El-Moatassim, 1993; Boarder et al., 1995), and chromaffin cells have been shown to respond to extracellular ATP by an increase in IP_3 formation and/or release of intracellular Ca^{2+} stores (Sasakawa et al., 1989; Castro et al., 1995). However, there are also a few reports that couple P_{2Y} receptors to an inhibition of adenylyl cyclase (Lin and Chuang, 1994). Because ATP does not elevate intracellular cAMP, it is unlikely it will recruit facilitation. It is also unlikely that the L-type channels will be inhibited by the same direct G protein interaction responsible for suppression of the N- and P/Q-type channels. Even if P_{2Y} receptor activation were to inhibit adenylyl cyclase in the chromaffin cells, an alternative pathway that recruits facilitation by PKA-independent phosphorylation exists (Artalejo et al., 1992b) and would presumably not be affected. Hence, under normal conditions, release of ATP from the chromaffin cells will tend to act primarily in a negative feedback manner to regulate the amount of secretion. With increased firing rates, there would be a tendency for this inhibitory role of ATP to persist and prevent excessive catecholamine release, but when the need for a large surge of catecholamine arises, the cells may still retain the capacity to overcome this inhibition through recruitment of facilitation Ca^{2+} channels.

More work is needed to substantiate these speculations, in particular the action (or lack of action) of ATP on facilitation Ca^{2+} channels and the dynamics and expression of P_{2X} as opposed to P_{2Y} purinoceptors. A better understanding of the temporal aspects of release and metabolism of ATP are needed. Nonetheless, the negative feedback pathway described in this manuscript may represent an important control mechanism for catecholamine secretion.

Experimental Procedures

Culture of Cells

Chromaffin cells were prepared by digestion of bovine adrenal glands with collagenase and purified by density gradient centrifugation as previously described (Artalejo et al., 1992a). The cells were plated on collagen-coated glass coverslips (22 × 22 mm) at a density of roughly 0.15×10^6 cells per cm^2 and maintained in an incubator at 37°C in an atmosphere of 92.5% air and 7.5% CO_2 with a relative humidity of 90%. Fibroblasts were effectively suppressed with cytosine-arabinoside (10 μM), leaving relatively pure chromaffin cell cultures. Although mixed, the cultures were somewhat enriched for epinephrine-containing over norepinephrine-containing cells. Half of the incubation medium was exchanged every day.

Electrophysiology

Chromaffin cells were voltage clamped in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) using an Axopatch-1C amplifier (Axon Instruments) at a holding potential of −80 mV, and peak inward I_{Ca} were activated by 20 ms step depolarizations to +10 or +20 mV. Prepulses were 100 ms step depolarizations to +100 mV. Current–voltage curves were generated by voltage ramps of 100 ms duration from the holding potential (−80 mV) to +100 mV. Leak currents were generated by averaging 16 hyperpolarizing sweeps (steps or ramps). All the data reported in this paper were capacitance and leak subtracted. The data were filtered at 2 kHz and then digitized at 100 μs per point. Series resistance was partially compensated (~80%) using the series resistance compensation circuit of the Axopatch-1C amplifier. Voltage protocols and data analysis were carried out in AxoBasic. Statistical significance was determined using paired or independent Student's t test.

Electrodes were pulled from microhematocrit capillary tubes (Drummond) and coated with sylgard (Dow Corning). After fire polishing, final electrode resistances when filled with the CsCl-based patch pipette solution (see below) were approximately 2 MΩ.

Solutions

Electrodes were filled with 110 mM CsCl, 4 mM $MgCl_2$, 20 mM HEPES, 10 mM EGTA, 0.35 mM GTP, 4 mM ATP, 14 mM creatine phosphate at pH 7.3 (adjusted by CsOH) and an osmolality of ~310 mOsm. In some experiments, the EGTA was lowered to 0.1 mM (and CsCl raised to 125 mM). Two extracellular solutions were used. The NaCl-based extracellular recording medium contained 140 mM NaCl, 2 mM KCl, 10 mM glucose, 10 mM HEPES, 2 or 10 mM $CaCl_2$, 0.7–1.0 μM tetrodotoxin (TTX) at pH 7.3 (adjusted with NaOH), and the osmolality was adjusted to 310 mOsm using sucrose. In a few experiments, the TTX was omitted. The TEA-based extracelluar solution contained 135 mM TEA-Cl, 10 mM glucose, 10 mM HEPES, 10 mM $BaCl_2$, 0.2 μM TTX at pH 7.3 (adjusted by TEA-OH), and the osmolality was adjusted to 310 mOsm using sucrose. Nisoldipine (1 μM) was present in all extracellular solutions to block any facilitation I_{Ca} (L-type). Neurotransmitters and agonists/antagonists were prepared as stock solutions in double-distilled water and diluted to the appropriate concentration in extracellular medium. In some cases, DMSO was used as a solvent to prepare stock solutions, but in final test solutions was present at very low (<0.05%) concentrations. Toxins (35–50 μL) were added directly to the bath (total volume 300–350 μL), with the flow of extracellular solution stopped at 10 times the desired final concentration. Thus, Cgtx was added at 10 μM to give a final concentration of ~1 μM, and Aga was added at 1 μM to give a final concentration of ~100 nM.

Stimulation of Endogenous Inhibition

The recording bath was designed so that it was small (<1 cm in diameter), and the volume of the bath was kept at around 300–350

μL. The bath solution was gravity fed from reservoirs. The TEA-based recording medium depolarized the non-voltage-clamped cells in the bath thereby stimulating secretion. The extracellular solution in the bath was either flowing (3–4 ml/min) or the flow was stopped. With the solution flowing, the material secreted by the chromaffin cells was rapidly washed away and so could not exert its actions. However, when the flow of the solution was stopped, the material secreted from the non-voltage-clamped cells accumulated rapidly and acted on the one voltage-clamped cell. Similar experiments were carried out in the NaCl-based recording medium, which also functioned as a control for perfusion artifacts. Agonists and antagonists were applied to the cells by including them in the recording solution and washing them into the bath. There was a latency between switching solutions at the reservoirs and the drugs reaching the cell due to "dead space" in the tubing leading to the bath. This accounted for the delay seen between agonist application and inhibition of I_{Ca} (for example see Figures 2 and 3). All experiments were carried out at room temperature (~23°C).

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